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(54) **Method for producing cells containing stably integrated foreign DNA at a high copy number, the cells produced by this method, and the use of these cells to produce the polypeptides coded for by the foreign DNA**

Verfahren zur Herstellung von Zellen, die stabil integrierte fremde DNS mit hoher Kopiezahl enthalten, durch dieses Verfahren hergestellte Zellen und Verwendung dieser Zellen zur Herstellung von durch diese fremde DNS kodierten Polypeptiden

Procédé de production de cellules contenant de l'ADN étranger, intégré de façon stable et à nombre de copies élevé, cellules produites par cette méthode et utilisation de ces cellules pour la production de polypeptides encodés par cer ADN

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(56) References cited:
EP-A- 0 281 246   WO-A-86/03780
US-A- 4 656 134

- **J. MOL. BIOL., vol. 189, 1986, pages 401-411, Academic Press Ltd, London, GB; F.A.M. ASSELBERGS et al.: "A recombinant Chinese hamster ovary cell line containing a 300-fold amplified tetramer of the hepatitis B genome together with a double selection marker expresses high levels of viral protein"**

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2.16.7/3.6

Description

<u>TECHNICAL FIELD OF THE INVENTION</u>

5   **[0001]** This invention relates to an improved method, employing electroporation, for producing recombinant host cells characterized by stably integrated foreign DNA at high copy number. These recombinant host cells are useful in the efficient, large-scale production of recombinant proteins and polypeptides.

<u>BACKGROUND OF THE INVENTION</u>

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[0002] The isolation of commercially useful quantities of polypeptides from their natural biological sources is often difficult and expensive. This difficulty is usually due to either a scarcity of source material or the presence of small amounts of polypeptide per unit of source material. Commonly, both of these factors operate simultaneously.

[0003] Advances in biotechnology have eased the production and isolation of polypeptides and proteins. A DNA
15   sequence coding for a desired polypeptide or protein may be inserted into a cloning vector along with appropriate regulatory sequences. The introduction of such a vector into a compatible host cell will result in production by the host cell of the polypeptide encoded by the inserted DNA sequence. Because of the cost of culturing cells and isolating the desired proteins from the cultures, the biotechnology industry has long sought methods to increase the yield of protein product produced per unit volume of transformed cell culture as well as the yield per unit time.
20   **[0004]** The level of production of a product by recombinant host cells is controlled by three major factors: (i) the number of copies of a DNA sequence coding for the product ("copy number") in the cell; (ii) the efficiency with which this DNA sequence is transcribed into messenger RNA; and (iii) the efficiency with which this messenger RNA is translated to generate the protein product. Efficiency of transcription and translation (which together comprise expression) is in turn dependent upon regulatory sequences located upstream and downstream of, and in some instances internal
25   to, the product gene.

[0005] None of the methods known in the art for introducing foreign DNA into eukaryotic cells results in stable integration of greater than approximately twenty copies of this foreign DNA into the host cell genome. Instead, these low copy number integrants must be treated to amplify their integrated foreign DNA, in order to produce high copy number integrants. The most widely used procedure to obtain high copy number integrants utilizes the dihydrofolate reductase
30   ("<u>DHFR</u>") gene.

[0006] Mammalian cells which contain multiple copies of the <u>DHFR</u> gene are selected when a culture of these cells is subjected to sequentially increasing concentrations of methotrexate (Alt et al., "Selective Multiplication of Dihydrofolate Reductase Genes in Methotrexate-Resistant Variants of Cultured Murine Cells", <u>J. Biol. Chem.</u>, 253, pp. 1357-79 (1978)). DHFR is an essential enzyme for cell survival. Since methotrexate ("MTX") is a competitive inhibitor of DHFR,
35   only those cells that have increased their DHFR content (e.g. by amplifying the resident <u>DHFR</u> gene) to offset MTX inhibition will survive. Furthermore, as the MTX concentration is sequentially increased, cells will require increasing amounts of DHFR, and thus higher <u>DHFR</u> gene copy numbers, to survive. This is the basis of the <u>DHFR</u> gene amplification procedure.

[0007] One indication that the <u>DHFR</u> gene might be useful in the amplification of cotransfected genes was the report
40   that when <u>Escherichia coli</u> plasmid pBR322 was cotransfected (introduced together) with genomic DNA containing a MTX-resistant <u>DHFR</u> gene into mouse cells, the pBR322 DNA was also amplified by MTX selection (Wigler et al., "Transformation of Mammalian Cells with an Amplifiable Dominant-Acting Gene", <u>Proc. Natl. Acad. Sci. USA</u>, 77(6), pp. 3567-70 (1980)). However, most of the Wigler transfectants did not amplify the pBR322 DNA more than several-fold.

[0008] The generation of very high copy number integrants was made possible by the isolation of Chinese hamster
45   cells deficient in native DHFR activity ("DHFR⁻ CHO cells") (Urlaub and Chasin, "Isolation of Chinese Hamster Cell Mutants Deficient in Dihydrofolate Reductase Activity", <u>Proc. Natl. Acad. Sci. USA</u>, 77(7), 4216-20 (1980). Transfection of these DHFR⁻ CHO cells with a plasmid containing both the <u>DHFR</u> gene and the <u>E. coli gpt</u> gene, followed by MTX selection, produced recombinant host cells which had amplified the <u>gpt</u> gene approximately 50-fold (Ringold et al., "Co-Expression and Amplification of Dihydrofolate Reductase CDNA and the <u>Escherichia coli XGPRT</u> Gene in Chinese
50   Hamster Ovary Cells", <u>J. Mol. Appl. Genet.</u>, 1, pp. 165-75 (1981)).

[0009] In a more dramatic example of the possibility for amplification of non-selectable genes using this technique, transfection of DHFR⁻ CHO cells with plasmids containing both the murine <u>DHFR</u> gene and the SV40 early region, followed by sequential step-wise increases in the MTX concentration of the growth medium, produced cells containing up to 1000 copies of the transforming DNA (Kaufman and Sharp, "Amplification and Expression of Sequences Cotrans-
55   fected with a Modular Dihydrofolate Reductase Complementary DNA Gene", <u>J. Mol. Biol.</u>, 159, pp. 601-21 (1982)).

[0010] While the DHFR/MTX amplification procedure produces cells with amplified copies of transfected DNA, it has several serious drawbacks. These drawbacks include the slowness of the procedure, the necessity of using DHFR⁻ cells to obtain significant amplification, and the fluidity of amplified DNA.

[0011] To select recombinant host cells which have amplified transfected DNA to a very high copy number, they must be subjected to sequential step-wise increases in the MTX concentration of the growth medium. This is a lengthy process. In our hands, six to ten months are required to achieve a several hundred-fold amplification. Obviously, a more expeditious procedure would be desirable.

[0012] Another drawback of the DHFR/MTX amplification procedure is that it does not work well for cells that contain a DHFR gene ("DHFR$^+$ cells"). At best, only a fifty-fold amplification of transfected DNA has been reported in DHFR$^+$ cells (Wigler et al., "Transformation of Mammalian Cells with an Amplifiable Dominant-Acting Gene", Proc. Natl. Acad. Sci. USA, 77(6), pp. 3567-70 (1980)). The production of DHFR$^-$ cells from DHFR$^+$ cells, if possible at all for a given cell type, is lengthy and laborious (Urlaub and Chasin, "Isolation of Chinese Hamster Cell Mutants Deficient in Dihydrofolate Reductase Activity", Proc. Natl. Acad. Sci. USA, 77(7), pp. 4216-20 (1980)). Since all mammalian cells possess the DHFR gene, a worker looking for significant amplification of transfected DNA would be restricted to using DHFR$^-$CHO cells unless he was willing to face the ordeal of creating a new DHFR$^-$ cell type.

[0013] An additional drawback of DHFR/MTX amplification is that not all sequences contained within transfected DNA will be amplified to the same degree (Kaufman and Sharp, "Amplification and Expression of Sequences Cotransfected with a Modular Dihydrofolate Reductase Complementary DNA Gene", J. Mol. Biol., 159, pp. 601-21 (1982)). Of equal concern are reports of deletions and rearrangements within amplified DNA (see, e.g., Kaufman and Sharp, id.; Schimke, "Gene Amplification in Cultured Animal Cells", Cell, 37, pp. 705-13 (1984)).

SUMMARY OF THE INVENTION

[0014] The present invention relates to a novel process, employing electroporation, for preparing recombinant host cells containing stably integrated foreign DNA which is at a high copy number.

[0015] A process of the present invention, as illustrated by one embodiment, is set forth below. An initial population of cells is transfected with DNA. This DNA comprises a gene coding for a desired polypeptide (the "product gene"). The transfecting DNA also comprises a "protective gene" that, on expression, codes for a protein in an amount capable of protecting the recombinant host cell against a toxic substance, wherein increasingly higher copy numbers of this gene are required to protect the recombinant host cell against increasing concentrations of this toxic substance. Alternatively, the protective gene may code, on expression, for a protein in an amount capable of protecting the recombinant host cell from decreases in the concentration of a previously essential nutritive substance. After electroporation, the cells are cultured for a time sufficient to allow these cells to recover from this transfection process, integrate foreign DNA at high copy number, and establish high level expression of the protective genes. Finally, the cells are subjected to a sufficiently high concentration of the toxic substance, or a sufficiently low concentration of the nutritive substance, that the protective gene was designed to protect against, killing or severely retarding the growth of those cells with a low copy number.

[0016] The process of this invention has numerous advantages over prior art methods and generally solves many of the problems associated with these methods.

[0017] Accordingly, it is an object of the present invention to provide a process which produces recombinant host cells containing stably integrated foreign DNA at a high copy number within a reasonable period of time. Another object of the present invention is that this process may successfully be applied to any eukaryotic cell.

[0018] These and other additional objects and advantages of the present invention are apparent from the detailed description and claims which follow.

[0019] In a preferred embodiment of the present invention, an initial population of cells is subjected to electroporation in the presence of foreign DNA. The foreign DNA comprises: (i) at least one protective gene coding for a protein capable of protecting the recombinant host cells against a toxic substance, wherein increasingly higher copy numbers of the protective gene are required for, and capable of, protecting the recombinant host cell against increasing concentrations of the toxic substance; (ii) at least one product gene coding for a polypeptide whose production is desired; and (iii) at least one selective gene coding for a protein, wherein the presence of one copy per cell of the selective gene is sufficient to protect the recombinant host cell against either decreases in the concentration of a nutritive substance or the presence of a toxic substance. These genes are operably linked to regulatory sequences that are compatible with the recombinant host cells.

[0020] Following electroporation, the cells are cultured for a time sufficient to allow them to recover from the electroporation process. After recovery, the cells are exposed to either: (i) a toxic substance against which the product of the selective gene provides protection, at a concentration sufficiently high to kill or severely retard the division of those cells which did not incorporate at least one copy of the foreign DNA in the electroporation process or (ii) a decreased amount of a nutritive substance which the product of the selective gene makes non-essential in the context of the selection scheme, for a time sufficient to kill or severely retard the division of those cells that did not incorporate at least one copy of the foreign DNA into their genomes after electroporation.

[0021] Finally, the cells are exposed to a sufficiently high concentration of the toxic substance, or a sufficiently low concentration of the nutritive substance which the protective gene protects against, killing or severely retarding the divi-

sion of those cells with a low copy number.

[0022] In another embodiment of the present invention, recombinant host cells are produced by two or more successive stages of electroporation and selection. Each stage of this plural stage process is performed as described above for a single stage process according to this invention, with the requirement that the foreign DNA added at each stage comprise a different protective gene.

[0023] In a still further embodiment of the present invention, recombinant host cells are produced by subjecting an initial population of cells to a single stage or plural stage process according to this invention, and then subjecting the recombinant host cells so produced to conventional amplification selection.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024]

Figure 1 is a pictorial representation of vector pSV2-DHFR.
Figure 2 is a pictorial representation of the construction of vector pJOD-10.
Figure 3 is a pictorial representation of vector pSV2-neo.

DETAILED DESCRIPTION OF THE INVENTION

[0025] The present invention relates to a novel process, employing electroporation, for preparing recombinant host cells containing stably integrated foreign DNA at a high copy number. For the purposes of this application, "high copy number" means at least fifty integrated copies of foreign DNA per recombinant host cell. Preferably, high copy number will be at least 100 copies per cell. Most preferably, high copy number will be 300 to 1000 copies per cell. For the purposes of this application, "low copy number" means less than about fifty copies of foreign DNA per recombinant host cell.

Cells

[0026] Recombinant host cells may be prepared, according to the method of this invention, from any eukaryotic cells. For example, cells of fungal, animal and plant origin would be appropriate. Host cells of animal origin are preferred for the production of polypeptides and proteins for use as pharmaceuticals and as medical diagnostic reagents. Useful animal cells may include B and T lymphocytes, leukocytes, fibroblasts, hepatocytes, pancreatic cells, and undifferentiated cells. Often, immortalized mammalian cell lines would be utilized. By way of example, useful mammalian cell lines would include 3T3, 3T6, STO, CHO, Ltk⁻, FT02B, Hep3B, AR42J and MPC1L. Preferred mammalian cell lines are CHO, 3T3, and Ltk⁻. While the frequency of stable transformants, and the expression level of transferred genes, will depend on the particular cell type used, the selection of a particular host is not part of this invention. Rather, this invention is applicable generally to the rapid production of recombinant cells containing stably integrated foreign DNA at a high copy number, from any eukaryotic cell.

Foreign DNA

[0027] The term "foreign DNA", as used in this application, refers to any DNA that is introduced into a host cell. This foreign DNA may be derived from a variety of sources. These sources include genomic DNA, cDNA, synthetic DNA and combinations thereof. If the foreign DNA comprises genomic DNA, it may include naturally occurring introns, located upstream, downstream, or internal to any included genes. Foreign DNA may also include DNA derived from the same cell line or cell type as the host cell, as well as DNA which is homologous or complementary to DNA of the host cell.

[0028] The foreign DNA will include at least one gene coding for a polypeptide or protein whose production is desired ("product gene") and at least one "protective gene", as defined infra. Preferably, the foreign DNA will also include at least one "selective gene", as defined infra. These genes may be contained in the foreign DNA within a single DNA molecule, within separate DNA molecules, or within combinations thereof.

[0029] The process of this invention is applicable to a wide variety of product genes and will result in cells with a high copy number of the desired product gene. This high copy number will usually result in high expression levels of the desired gene product. For example, products such as cellular receptors, the active components of vaccines or other pharmaceutically active products, agriculturally or other commercially useful compositions, enzymes, antibiotics, food stuffs and the like are usefully produced by the recombinant host cells produced by the process of this invention. Some specific examples of desirable product genes are those coding for tissue plasminogen activator, Müllerian inhibiting substance, factor VIII, erythropoietin and soluble CD4. Again, the selection of a particular product gene is not a part of this invention. Rather, this invention is generally applicable to producing recombinant host cells with high expression

levels of desired gene products due to the high copy number of product genes in the recombinant host cell.

[0030] As used in this application, a "protective gene" is a gene that, on expression, is capable of protecting the recombinant host cell from a toxic substance, where increasingly higher copy numbers of this protective gene are required to protect the recombinant host cell against increasing concentrations of the toxic substance. Alternatively, the
5   protective gene may code for a protein that is capable of protecting the recombinant cell from decreases in the concentration of a previously essential nutritive substance in the culture medium, where increasingly higher copy numbers of the protective gene are required to protect the recombinant host cell against decreasing concentrations of the nutritive substance.

[0031] In general, any gene that is amplified when the host cell containing it is subjected to suitable selection pressure
10 will serve as a protective gene for the purposes of the present invention. Thus, a great variety of protective genes may be utilized, providing that, at the level they are expressed, their gene products are capable of protecting the host cell from a toxic substance or the lack of a nutritive substance, and, providing that the degree of protection these gene products confer is roughly proportional to the copy number of the protective gene. For example, if the product of a protective gene protects the cell from a toxic substance, then increasingly higher copy numbers of this gene are required to protect
15 the recombinant host cell from increasing concentrations of the toxic substance that the protective gene was designed to protect against. In the context of this application, "protection" of the recombinant host cells means that cell division is not severely inhibited. A failure of protection would thus indicate either the death of the cells or a severe inhibition of cell division. There are three broad classes of protective genes.

[0032] The first class of protective genes includes those genes that confer the desired protection on their host cell
20 (i.e., allow for selection of cells comprising high copy number foreign DNA) even when expressed from efficient promoters. Examples of this type of protective gene and the toxic substances these genes protect against include: dihydrofolate reductase (methotrexate), metallothionein (heavy metals, e.g., cadmium), CAD (N-(phosphonacetyl)-L-aspartate), 3-hydroxy-3-methylglutaryl coenzyme A reductase (compactin), adenosine deaminase (deoxycoformycin-alanosine-adenosine-uridine), uridine monophosphate synthetase (pyrazofurin), glutamine synthetase (methionine sul-
25 foxine ir glutamine-free media), asparagine synthetase (β-aspartylhydroamate), ribonucleotide reductase (hydroxyurea), thymidilate synthetase (5-fluoro-deoxyuridine), ornithine decarboxylase (difluoromethylornithine), and promoter glutathione S-transferase Ya (benzo(α)pyrene anti-diol epoxide). Examples of this type of protective gene and the nutritive substances whose reduction or absence they protect against include: thymidine kinase (thymidine) and xanthine-guanine phosphoribosyl transferase (xanthine).

30 [0033] The second class of protective genes includes those dominant selectable marker genes that, when expressed from efficient promoters, confer the desired protection on their host cell (i.e., allow for selection of cells comprising high copy number foreign DNA) by virtue of point or deletion mutations that greatly impair the ability of their gene products to protect the host cell from selection pressure. Thus, a typical selective gene, whose gene product at normal expression levels is so active that the host cell requires only one gene copy to survive a wide range of selection pressure, may
35 be "converted" to a protective gene. Such a "conversion" would be accomplished by mutating the selective gene so that the ability of its gene product to protect the host cell from selection pressure is substantially impaired. The host cell of such a mutated gene would then require increasingly higher copy numbers of this mutated gene to survive increasingly stringent selection pressure.

[0034] The third class of protective genes includes those selective genes that confer the desired protection on the
40 host cell by virtue of the fact that they are expressed at extremely low levels. This low expression level may be achieved either by drastically reducing translation or transcription. One way to effectively reduce translation would be to situate the selective gene so that it constitutes the second coding region of a dicistronic messenger RNA. To effectively inhibit transcription of the selective gene, it may be coupled to a profoundly defective, or inoperable, promoter. Alternatively, all but the TATA box of the promoter may be deleted. By severely inhibiting expression of the selective gene, it is, in
45 effect, being functionally converted to a protective gene, so that a single copy of this gene is no longer sufficient to protect the host cell over a wide range of selection pressure. Rather, increasingly higher copy numbers of this gene are required to protect the host cell from increasingly stringent selection pressure.

[0035] In a preferred embodiment of this invention, the foreign DNA will also include at least one "selective gene". As used in this application, a "selective gene" is a gene (dominant selectable marker) coding for a protein that is capable
50 of protecting the recombinant host cell from either the presence of a toxic substance or the absence of a nutritive substance, where the presence of only one copy of the selective gene is sufficient to protect the recombinant host cell over a wide range of selection pressure. Examples of useful selective genes include: neo (G418 resistance), gpt (xanthine utilization in the presence of mycophenolic acid), hisD (histidinol utilization), and hygro (hygromycin B resistance).

[0036] Except for the third class of protective genes noted above, each protective, selective and product gene must
55 be operably linked to a 5'-noncoding DNA sequence comprising transcriptional and translational initiation elements which are compatible with the particular host cell utilized. If two or more genes share the same promoter/enhancer elements (resulting in a multicistronic mRNA), the product gene should be closest to the promoter in order to optimize its expression level. However, it is preferable to link each gene to its own regulatory region. In general, 5'-regulatory

sequences will be chosen to maximize expression of the transferred genes, especially product genes.

[0037]   A wide variety of transcriptional and translational 5'-regulatory sequences may be successfully employed. The transcriptional regulatory sequences must include promoter elements and may include enhancer elements. The various 5'-regulatory elements may be derived from one source, or a combination of sources. If a gene is supplied as genomic

5   DNA, the regulatory sequences normally associated with that gene may be utilized. Alternatively, these regulatory elements may be derived from the 5'-non-coding region of other genes. For example, if a mammalian host cell is utilized, these 5'-regulatory elements may be derived from viruses such as adenovirus, bovine papilloma virus, Simian virus 40, cytomegalovirus, or the like, where these sequences are associated with a gene that has a high level of expression. Alternatively, the 5'-noncoding regions of eukaryotic genes, such as β-actin, with high levels of expression may be used.

10   **[0038]**   It may be advantageous to utilize transcriptional initiation regulatory sequences which allow for repression and activation, so that expression of a particular gene may be modulated at will. Although it will normally be desirable to optimize expression of the transferred genes through the choice of particular 5'-regulatory sequences, so long as the transferred genes are expressed, the choice of 5'-regulatory sequences is not critical. Appropriate 5'-regulatory sequences for a particular host cell may conveniently be chosen from among the available 5'-regulatory sequences that are known

15   in the art to be compatible with that cell type.

[0039]   Normally, there are 3'-regulatory sequences operably linked to the transferred genes. For example, a poly A addition site is usually positioned downstream of every transferred gene. If the gene is supplied as genomic DNA from a eukaryotic source, the poly A site naturally associated with that gene may be used. Alternatively, the poly A addition sequence of another gene may be employed. This sequence may be contiguous with the transferred gene. Alterna-

20   tively, the poly A site may be placed further downstream of the gene, either with or without a splice junction.

[0040]   In a preferred embodiment of this invention, at least one eukaryotic transcriptional terminator element will be positioned downstream of every protective, selective, and product gene. If a gene has an associated poly A addition site, then the terminator element will be positioned downstream of this poly A addition site. The use of such transcriptional terminator elements resulted in an approximately four-fold increase in expression of the product gene.

25   **[0041]**   While not wishing to be bound by theory, we believe that the presence of terminator elements downstream of these genes results in improved expression levels by decreasing transcriptional interference between the genes.

[0042]   If the foreign DNA consists of plasmid DNA and more than one gene is on each plasmid, then the promoter region of each gene will likely not be very far away from the end of an adjacent gene. Therefore, an RNA polymerase molecule transcribing a gene may continue to progress along the DNA strand past the end of that gene and into the

30   promoter region of the next gene, interfering with the transcription of this downstream gene. If, however, a transcriptional terminator element is positioned between the two genes, the RNA polymerase will not reach the promoter of the downstream gene and will thus not interfere with its transcription. Accordingly, it is especially preferred that a transcriptional terminator element be placed upstream of any product gene to maximize its expression. However, it is not essential that every selective or protective gene be preceded by a terminator element.

35   **[0043]**   Examples of useful eukaryotic transcriptional terminator elements include the human gastrin gene terminator (Sato et al., "A Specific DNA Sequence Controls Termination of Transcription in the Gastrin Gene", Mol. Cell. Biol., 6, pp. 1032-43 (1986)) and the sea urchin H2A histone gene termination sequence (Johnson et al., "Tripartite Sequences within and 3' to the Sea Urchin H2A Histone Gene Display Properties Associated with a Transcriptional Termination Process", Mol. Cell. Biol., 6, pp. 4008-18 (1986)). However, this invention should not be construed as being limited to

40   these terminator elements. Rather, any sequence which becomes known in the art as having the ability to halt the progression of RNA polymerase II would be appropriate for the purposes of this invention. Preferably, any terminator element used will be positioned in its natural orientation with respect to the gene whose transcription it is to terminate.

[0044]   In addition to the regulatory elements discussed above, other transcriptional and translational control elements located either within or without the transcribed regions of the genes may be utilized to good advantage. In addition,

45   sequences which increase the frequency of chromosomal integration or recombination and sequences which contain origins of replication may be useful. Among elements located within the transcribed regions of the genes, sequences which enhance RNA processing and export from the nucleus as well as those which stabilize the mRNA and increase its translation may be useful.

[0045]   The physical state of the foreign DNA is important to its efficient integration into the host cell genome at a high

50   copy number. In the preferred embodiment of this invention, the input foreign DNA will be linear. Supercoiled or relaxed circular DNA is much less preferred. In addition, to practice the preferred embodiment of this invention, the linear input foreign DNA will have compatible cohesive ends. The use of linear foreign DNA with cohesive ends results in a 5- to 20-fold greater yield of high copy number integrants than the use of linear DNA with blunt ends. Cohesive ends (also called "sticky ends" in the art) are created by digesting the foreign DNA with a restriction enzyme that makes a staggered cut,

55   resulting in linear DNA in which one strand "overhangs" the other. While we prefer an overhang of at least four base pairs, both longer and shorter overhangs are also useful for the purposes of this invention.

[0046]   If the input foreign DNA consists of more than one type of DNA molecule (e.g., if the protective gene is on one plasmid and the product gene is on another), each type of DNA molecule should be digested with enzymes that result

in linear DNA molecules with cohesive ends that are compatible ("cohesive") with all other DNA molecules in the foreign DNA. While not wishing to be bound by theory, we believe that cohesive ends improve the efficiency of high copy number integration because the foreign DNA molecules may ligate in tandem to form a large DNA molecule and that it is this large DNA molecule that then integrates into the genome. Thus, a single integration event may produce a high
5 copy number integrant.

[0047] As stated above, foreign DNA may consist of genomic DNA, synthetic DNA, cDNA or any combination thereof. In the preferred embodiment of this invention, the foreign DNA will consist of plasmid DNA which further comprises sequences allowing for plasmid replication and selection in bacteria. The use of such plasmids is preferred because they permit convenient production of large quantities of pure foreign DNA. The particular plasmid utilized is not critical
10 to the practice of this invention. Rather, any plasmid known in the art to be capable of being replicated and selected for in bacteria would be a suitable vehicle in which to insert desired product, selective and protective genes, as well as their attendant regulatory sequences.

[0048] Each protective, selective and product gene may be contained within a separate DNA molecule. However, in the preferred embodiment of this invention, any product gene will be contained within the same DNA molecule as at
15 least one protective gene. If a selective gene is employed then it is preferable to include this gene on the same DNA molecule as the protective and product genes.

[0049] If the foreign DNA consists of DNA molecules that each contain more than one gene, and each of these genes is followed by a transcriptional terminator element, then spacing and orientation of the genes is probably not critical. However, if transcriptional terminator elements are not present, then care ought to be exercised as to the spacing and
20 orientation of the genes within each DNA molecule. A back-to-back orientation of genes is preferred over a head-to tail orientation. If two genes are in a back-to-back orientation, there should be at least 1000 base pairs between their respective poly A sites. If two genes are in a head-to-tail orientation, at least 2000 base pairs should separate the poly A site of the upstream gene from the promoter of the downstream gene.

25 Electroporation

[0050] In order to produce high copy number integrants, the foreign DNA must be introduced into the host cells by electroporation. Preferably, an electroporation protocol which results in efficient DNA transfection (transient introduction of at least about 2000 copies of foreign DNA per cell) would be utilized. In the preferred embodiment of this invention,
30 we use the protocol set forth below. This protocol is a modification of that presented by Chu et al., "Electroporation for the Efficient Transfection of Mammalian Cells with DNA", 15(3), pp. 1311-27 (1987). However, this invention does not exclude alternative electroporation protocols which also result in efficient transfection (i.e., those that result in transient introduction of at least about 2000 copies of foreign DNA per cell).

[0051] Preferably, the cells will be fed or passaged on the day prior to electroporation. In addition, at the time the cells
35 are harvested for electroporation, they should be growing exponentially. If monolayer cells are used, they should be one-half confluent. During electroporation, the cell density should be from 5×10^6 to 3×10^7 cells/ml. Preferably, the cell density would be 1.25×10^7 cells/ml.

[0052] A high DNA concentration during electroporation will increase the efficiency of transfection. It will also increase the total number of integration events, and the frequency of high copy number integration events. Preferably, the DNA
40 concentration (including carrier DNA) will be 0.5 mg/ml.

[0053] The inclusion of carrier DNA results in a significant improvement in the yield of transformants and high copy number integrants. Preferably, the carrier DNA will be approximately 300 to 1000 bp in length. Carrier DNA significantly larger than 1000 bp is toxic to the cells. Sonication may conveniently be used to produce carrier DNA with the desired size distribution. Any genomic DNA would be appropriate for use as carrier. Preferably, salmon sperm DNA would be
45 utilized because of its ready commercial availability in large quantities at reasonable prices. The ratio of foreign DNA to carrier DNA may vary, but the total concentration of DNA is maintained as above. Typically, foreign DNA will comprise 30 to 80% of the total DNA, and preferably 37 to 50%.

[0054] The foreign and carrier DNAs are added to the cells and the resulting mixture transferred to the container in which electroporation is to occur. It is not necessary to preincubate cells and DNA prior to electroporation.
50 [0055] We prefer to perform electroporation in a buffer of the following composition: 20 mM Hepes/NaOH, pH 7.05; 137 mM NaCl; 5 mM KCl; 0.7 mM Na_2HPO_4; 6 mM dextrose. However, other buffers approximating physiological conditions would be acceptable.

[0056] Preferably, electroporation will be carried out at about 25°C. Electroporation at 0-4°C was five- to twenty-fold less efficient in terms of the yield of primary transformants and high copy number integrants.
55 [0057] A Bio-Rad Gene Pulser™ electroporation apparatus was utilized in the preferred embodiment. However, a different apparatus generating the same waveform would give substantially the same results. The Gene Pulser™ apparatus generates an exponential decay pulse by discharge of a capacitor. The characteristics of the exponential decay waveform will be determined by the following variables: (a) the amount of charge the capacitor will store at a given volt-

age (usually stated in microfarads), (b) the voltage to which the capacitor is charged, and (c) the resistance of the circuit through which the capacitor is discharged (including the resistance offered by the electroporation buffer by virtue of its volume and ionic strength). Accordingly, it is these variables that must be optimized to achieve the desired results.

[0058] Typically, electroporation was performed in 800 µl of buffered saline (the exact buffer composition is set forth above). This is the capacity volume of the Gene Pulser™ electroporation cuvette. The capacitor was set to the maximum capacitance that the apparatus would allow (960 µF). The Gene Pulser™ apparatus will produce a nearly uniform electrical field of strength $E = V/0.4$ cm. The optimal voltage will vary depending upon the cell type; if the voltage is too high, the cells will die. In general, the optimal voltage for a particular cell type will be between about 230 - 320 V, with the other variables as set forth above. It is a sharp optimum, straying as little as 10 V away from the optimum will result in a significantly lower efficiency of DNA uptake by the cells. A single pulse, lasting approximately 10 milliseconds, is delivered to the contents of the cuvette.

[0059] After the pulse, the cells are allowed to stand for a brief rest period (usually 8 - 10 minutes) before removal from the cuvette. Typically, approximately 20 to 50% of starting cells survive the electroporation process.

Selection

[0060] In the preferred embodiment of the present invention, the foreign DNA will include a selective gene, in addition to product and protective genes. In this instance, the cells would be subjected to both a primary, and a secondary selection step. In the primary selection step, cells that have integrated at least one copy of the selective gene are selected. For the primary selection step to work, the selective gene must be expressed. After the secondary selection step, 50 - 80% of the surviving clones are characterized by high copy number integration of foreign DNA.

[0061] Survival at a given selection pressure in secondary selection is not necessarily directly proportional to gene copy number. The location in the host cell genome at which the foreign DNA integrates can have a significant effect on its transcription. Thus, "position effects" will influence the expression level per gene copy. In addition, mutant cells that have an impaired ability to take up the toxic substance, or an improved uptake of the nutritive substance, that the protective gene was designed to protect against, might be able to survive secondary selection without high copy number integration. Finally, it is possible that the protective gene may mutate so that its gene product is resistant to the toxic substance it was designed to protect against. However, these would be rare events. The majority of cells surviving secondary selection are characterized by high copy number integrated foreign DNA.

[0062] The timing of the selection steps is important to their success and is set forth below. After the cells are removed from the electroporation cuvette, they are seeded into culture plates in non-selective medium. The cells are cultured in non-selective medium for a time sufficient to allow them to recover from electroporation and express the selective gene. Usually, an incubation of two to four days (or generation times) in non-selective medium is required prior to primary selection. Preferably, an incubation of two days (or generation times) is performed.

[0063] Next, the cells are subjected to primary selection by culturing them in medium (i) that is supplemented with the toxic substance that the selective gene was designed to protect against or (ii) that is depleted in the nutritive substance that the selective gene makes non-essential in the context of the selection scheme utilized. The particular toxic or nutritive substance chosen, as well as the appropriate concentration of that substance for primary selection, depends upon which selective gene and selection scheme is utilized, and can be chosen according to standard criteria.

[0064] The primary selection step will preferably last four days (generation times). However, primary selection periods of four to six days (or generation times) would be appropriate. The primary selection step either kills or severely retards the division of those cells that are not expressing at least one copy of the selective gene.

[0065] The secondary selection step should not be commenced earlier than six days (or generation times) after electroporation, nor later than nine days (or generation times) after electroporation, irrespective of the durations of the non-selective and primary selection incubations. Normally, the secondary selection step would commence seven days (or generation times) after electroporation. While not wishing to be bound by theory, we believe that this lag period is required to allow recovery from electroporation, integration of high copy number foreign DNA and establishment of stable high level expression of the protective genes. In a secondary selection, the cells are cultured in medium (i) that is supplemented with the toxic substance that the protective gene was designed to protect against or (ii) that is depleted in the nutritive substance that the protective gene makes non-essential. The secondary selection step either kills or severely retards the division of those cells that are not expressing large amounts of the protective gene product.

[0066] The appropriate concentration of toxic or nutritive substance may be conveniently determined as follows. First, a concentration at which at least 80% of the primary transformants survive should be determined. Next, the cells' resistance to a range of concentrations above that level should be determined, with increases of approximately two-fold between concentrations. This range should include at least one concentration at which no colonies are obtained (survival frequency of less than 10^{-7}). The appropriate concentration will be that at which the survival frequency is within the range of approximately 5×10^{-6} to 1×10^{-7}.

[0067] In an alternative embodiment of the present invention, the foreign DNA will not include a selective gene. In this

case, no primary selection step would be performed. After electroporation, the cells are cultured in non-selective medium prior to commencing secondary selection. The appropriate time to commence secondary selection is as described in the previous embodiment.

[0068]   Once secondary selection has been commenced, the cells must thereafter be cultured in secondary selection medium in order to maintain high copy number. Usually, when colonies large enough to subclone (~1 mm in diameter) have grown up, they are picked and separately expanded. The time required after the commencement of secondary selection until colonies large enough to subclone have grown up depends on the generation time of the particular cell line used and is not critical to practicing the invention. In general, however, clonal cell populations containing foreign DNA at high copy number will be produced by the process of this invention within twenty one days after electroporation.

[0069]   Alternatively, cells need not be subcloned after secondary selection. If subcloning is not performed, then the resulting cell population would contain cells of varying genotypes. However, the majority of these cells will contain foreign DNA integrated at high copy number.

Recombinant Host Cells

[0070]   Fifty to eighty percent of the recombinant host cells produced by the process of this invention are characterized by stably integrated high copy number foreign DNA.

[0071]   In cells containing high copy number DNA, produced by traditional gene amplification techniques, the individual repeating units are usually very large, and may range in size from hundreds to thousands of kilobase pairs (see, e.g., Roberts et al., "A Structure for Amplified DNA", Cell, 33, pp. 53-63 (1983); Dolnick et al., "Correlation of Dihydrofolate Reductase Elevation with Gene Amplification in a Homogeneously Staining Chromosomal Region in L5178Y Cells", J. Cell Biol., 83, pp. 394-402 (1979)). Individual units contain variable amounts of flanking cellular DNA and/or carrier DNA. Consequently, the repeating units within a particular tandem array are highly heterogeneous in terms of both size and sequence content (see, e.g., Roberts et al., id.; Schimke, "Gene Amplification in Cultured Animal Cells", Cell, 37, pp. 703-13 (1984)).

[0072]   In contrast, greater than 70% of the high copy number clones produced have foreign DNA copies inserted into the host cell genome in the form of tandem or inverted tandem repeats, with each repeating unit apparently containing only foreign DNA. In these tandem repeat clones, most, but not all of the foreign DNA copies are found in these tandem repeats.

[0073]   In approximately 80% of these tandem repeat clones, the restriction site used to originally linearize the input foreign DNA was still intact. This result indicates that the occurrence of these high copy number clones is probably due to ligation of the input DNA followed by integration as tandems, rather than by integration of single units followed by gene amplification. Approximately 20% of these clones had tandem repeats in which the original restriction sites had been destroyed by nuclease degradation of 20-200 base pairs at each end.

[0074]   If all the protective, product and selective genes are contained within the same DNA molecule, then each repeating unit will be essentially identical. When the genes are contained on two or more DNA molecules, then the repeating units will reflect this diversity. If there are different types of foreign DNA repeating units then they will be randomly ligated to form the tandems. In either situation, the orientation of individual units will be random.

[0075]   In approximately 10 - 20% of the recombinant host cells, the high copy number foreign DNA is present within the genome as scattered single site single copy, or low copy tandem, insertions.

[0076]   In approximately 5 - 10% of the recombinant host cells of this invention, the structure of the integrated copies of foreign DNA was not experimentally discernable.

[0077]   The copy number of the high copy number clones analyzed was stable for at least 150 generations.

Plural Stage Electroporation

[0078]   In another embodiment of the present invention, recombinant host cells comprising foreign DNA integrated at high copy number are prepared by a plural stage electroporation process.

[0079]   The first stage of a plural stage electroporation process according to this invention would be conducted as described supra. The high copy number recombinant host cells obtained after the first stage of electroporation and selection would then be subjected to a one or more further stages of electroporation and selection as described supra, with the requirement that the foreign DNA added at each stage comprise a different protective gene from that used in any other stage. A two stage process is preferred.

[0080]   Generally, the foreign DNA used at each stage of a particular plural stage process will comprise the same product gene. However, it may be desirable to utilize foreign DNA comprising different product genes in different stages.

[0081]   By way of example, in a two stage electroporation process according to this invention, the protective gene utilized in the first stage might be the dihydrofolate reductase gene, and that used in the second stage might be the adenosine deaminase gene.

Selection Followed By Amplification

[0082] If the recombinant host cells of the previous embodiments are cultured for long periods of time in secondary selection medium, with increasing selection pressure, then at least some of these cells will amplify their complement of foreign DNA by conventional gene amplification. The amplified recombinant host cells so produced would have an even higher foreign DNA copy number than the original high copy number integrants. Presumably, these cells would also have higher expression levels of foreign DNA genes.

[0083] In a preferred method of this embodiment, such amplified high copy number recombinant host cells ("amplified daughter cells") are produced by culturing high copy number recombinant host cells ("parent cells"), the high copy foreign DNA of which comprises the dihydrofolate ("DHFR") gene as the protective gene, at low density in an amplification selection medium comprising methotrexate ("MTX") at a concentration that is greater than the maximum MTX survival concentration of the parent cells.

[0084] To determine the maximum MTX survival concentration of a particular parent cell clone, an MTX killing experiment is performed as follows. The parent clone is seeded at a density of 10^3 to 10^5, and preferably 10^4, cells per 10 cm tissue culture plate in culture medium comprising MTX at a concentration equal to that present during the secondary selection step that produced the parent clone. In other plates, the parent clone is seeded at the same density in culture media comprising incrementally higher concentrations of MTX, up to 5 to 20 μM MTX, and preferably up to 10 μM MTX. Preferably, two-fold increments of MTX will be tested. After 5 to 8 days, and preferably 7 days, the surviving cells in each plate are counted. From these survival frequencies, the maximum MTX survival concentration is determined. It is the concentration that resulted in at least 50% as many cells surviving as those surviving at the MTX concentration that was present during secondary selection of the parent clone.

[0085] The amplification selection medium of a particular parent clone will comprise MTX at a concentration greater than the maximum MTX survival concentration of that clone, determined as described above. Preferably, the amplification selection medium will comprise MTX at a concentration that is 2 to 10 fold greater than the maximum MTX survival concentration of that parent clone. Most preferably, the MTX concentration will be three fold greater than the maximum MTX survival concentration. Prudence would recommend that a parent clone be subjected to amplification selection at several different MTX concentrations, chosen as described above.

[0086] The parent cells are cultured in amplification selection medium for 10 to 16 days to obtain cells (amplified daughter cells) that have amplified the high copy number DNA of the parent clone and produce higher levels of the polypeptide encoded by the product gene.

[0087] The frequency with which the above amplification selection protocol causes parent (high copy number) clones to give rise to amplified daughter clones is highly variable. Indeed, approximately 25% of high copy number parent clones were refractory to amplification, giving rise solely to unamplified daughter cells that did not produce higher levels of the polypeptide encoded by the product gene. In addition, there appears to be an inverse relationship between the maximum MTX survival concentration of a parent clone and the frequency of amplified daughter clones produced from that parent clone. We do not as yet know the cause of this apparent inverse relationship.

[0088] A possible explanation for the apparent inverse relationship between maximum MTX survival concentration and the frequency of amplified daughter clones is that as the MTX concentration is increased to higher levels the proportion of daughter clones that survive due to decreased MTX uptake, rather than increased DHFR gene copy number, is increased. Such a phenomenon, if it occurs, may likely be circumvented by utilizing parent cells which comprise high copy number foreign DNA that has been altered to reduce expression of DHFR while maintaining the same expression level of the product gene. This approach should allow amplification selection of cells with the same foreign DNA copy numbers at lower MTX levels.

[0089] One method of modifying foreign DNA to reduce expression of DHFR while maintaining the expression level of the product gene would be to drive transcription of the DHFR gene with a promoter that is relatively weak. In particular, it should be weaker than that used to drive transcription of the product gene. For example, the SV40 promoter with deletions of either or both of its enhancers --nucleotides 200-270 and/or 128-200 (nucleotide designations according to SV40 sequence) -- or the thymidine kinase promoter might be used to drive DHFR expression.

[0090] Another way to reduce DHFR expression would be to insert a short AT-rich segment comprising at least one copy, and preferably three or more copies, of the sequence ATTTA into that part of the DHFR gene that codes for the 3'-untranslated region of DHFR mRNA. The presence of an AU-rich segment, comprising copies of the AUUUA sequence, in the 3'-untranslated region of an mRNA is thought to significantly reduce the half-life of the mRNA which contains it. See, e.g., Clemens, "A Potential Role for RNA Transcribed from B2 Repeats in the Regulation of mRNA Stability", Cell, 49, pp. 157-58 (1987); Shaw and Kamen, "A Conserved AU Sequence from the 3' Untranslated Region of GM-CSF mRNA Mediates Selective mRNA Degradation", Cell, 46, pp. 659-67 (1986). Thus, inserting such a segment after the coding region of the DHFR gene would decrease the expression level of DHFR.

[0091] The AU-rich segment would be effective at any location within the 3'-noncoding sequence of the DHFR mRNA. For example, the AT-rich segment may be inserted into the BglII site at the junction between the end of the DHFR gene

and the SV40 sequences of vector pSV2-DHFR (Figure 1) or pJOD-10 (Figure 2).

[0092]   Many AU-rich segments comprising one or more copies of the AUUUA sequence would be useful for the purpose of the present invention. Segments comprising three or more copies of the AUUUA sequence are preferred. For example, the AU-rich segments found in the 3'-untranslated regions of the various human and murine mRNAs recited
5   at page 157 of Clemens, supra, would be useful. A preferred AT-rich segment is the following 54-nucleotide sequence found in the 3'-untranslated region of the murine GM-CSF gene:

ATATTTATTTATATATTTATATTTTTTAAATATTATTTATTTATTTATTTATTT

10

That sequence is recited in Figure 2A, at page 660 of Shaw and Kamen, supra. The corresponding sequence (see id.) of the human GM-CSF gene is also preferred.

[0093]   Based on published reports and on our experience, the maximum foreign DNA copy number that may be obtained using conventional step-wise DHFR/MTX amplification appears to be approximately 1000 copies per cell. In
15   contrast, this embodiment of the present invention -- high copy number electroporation followed by amplification -- has yielded clones possessing 2000-5000 copies of foreign DNA. Thus, the process of this embodiment allows production of clones with higher product gene expression levels (due to the greater gene copy number) than would be possible using conventional step-wise DHFR/MTX amplification. And the process of this embodiment requires less time than conventional step-wise amplification.

20   [0094]   While not wishing to be bound by theory, we believe that the process of this embodiment results in greater foreign DNA copy numbers than that possible by conventional step-wise amplification for the following reason. When conventional step-wise amplification increases one gene copy to 200-1000 copies, each amplification unit (one gene usually) is contained in an amplification domain of 100,000 to 500,000 base pairs. As high copy numbers are obtained, huge amounts of new DNA are added to the chromosome containing the amplified DNA. The amplified DNA is usually
25   seen to reside in a homogeneously staining region ("HSR") of the chromosome. This HSR can be larger than the size of an average chromosome. Therefore, the gene copy number may be limited by the size of a chromosomal HSR unit which can be carried. In the process of this embodiment of the present invention, each amplification unit contains many gene copies, usually in tight tandem arrays. At a given copy number, therefore, the "DNA burden" would be far less.

[0095]   Amplification selection of high copy number parent cells comprising protective genes other than DHFR may be
30   accomplished analogously to the procedure outlined above for DHFR/MTX amplification selection.

[0096]   In order that this invention may be more fully understood, the following Examples are set forth. These Examples are for purposes of illustration only and this invention should not be construed to be limited by any recitation used therein.

35   ## EXAMPLE 1

Effect of Methotrexate Concentration During Secondary Selection on DHFR Gene Copy Number in CHO Cells

Cells and Media

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[0097]   DHFR⁻ CHO cells were subcloned from the clone designated CHO-DUKX-B1of Urlaub and Chasin, "Isolation of Chinese Hamster Cell Mutants Deficient in Dihydrofolate Reductase Activity", Proc. Natl. Acad. Sci. USA, 77, pp. 4216-20 (1980). This clone was kindly provided by Dr. P. A. Sharp (Massachusetts Institute of Technology, Boston, MA), who obtained the clone from Dr. L. Chasin (Columbia University, New York, NY). The cells were grown in MEM alpha
45   supplemented with ribonucleotides and deoxyribonucleotides (10 mg/L each of adenosine, cytidine, guanosine, uridine, 2'-deoxyadenosine, 2'-deoxyguanosine and 2'-deoxythymidine; 11 mg/L of 2'-deoxycytidine hydrochloride) (Gibco Laboratories, Grand Island, NY), 10% fetal bovine serum (Hazleton, Lenexa, KS) and 4 mM glutamine (M.A. Bioproducts, Walkersville, MD) ("α⁺ medium").

[0098]   For primary selection, DHFR⁻ CHO cells were transferred to MEM alpha supplemented with 10% dialyzed fetal
50   bovine serum (Hazleton) and 4 mM glutamine and lacking ribonucleotides and deoxyribonucleotides ("α⁻ medium").

[0099]   For secondary selection, the cells were cultured in α⁻ medium supplemented with methotrexate ("α⁻/MTX medium") (Sigma Chemical Co., St. Louis, MO) (see Table I or II for the various MTX concentrations tested).

Expression Vectors

55

[0100]   The vector pSV2-DHFR (Figure 1) expresses DHFR from the SV40 early promoter. The construction of this vector is described in Subramani et al., "Expression of the Mouse Dihydrofolate Reductase Complementary Deoxyribonucleic acid in Simian Virus 40 Vectors", Mol. Cell. Biol., 1(9), pp. 854-64 (1981). Vector pSV2-DHFR, harbored in E.

coli strain HB101, is available from the American Type Culture Collection, Rockville, Maryland (ATCC 37146).

Electroporation and Selection of High Copy Number CHO Cells

[0101] This example summarizes the results of two separate experiments (i.e., selection of high copy number cells from two independent electroporations). Both of these experiments were performed identically, and are described below. Unless otherwise noted, all operations were performed at room temperature.

[0102] Foreign DNA was prepared for transfer into host cells as follows. Two hundred micrograms of the vector pSV2-DHFR were digested overnight at 37°C with EcoRI to linearize the DNA (400 µl reaction containing 200 µg DNA and 100 units EcoRI). After digestion, 200 µg of salmon sperm DNA, previously sonicated to 300-1000 bp size, were added to the linearized vector and the mixture of DNAs was precipitated. To precipitate, 5 M NaCl was added to a final concentration of 0.1 M. Next, 2.5 volumes of ethanol were added, and the mixture incubated for ten minutes on dry ice. After a ten minute centrifugation at 4°C in an Eppendorf centrifuge (model 5414), the ethanol was aspirated and the DNA pellet was air-dried for 15 minutes in a tissue culture hood. Eight hundred microliters of 1X HeBS (20 mM Hepes/NaOH, pH 7.05; 137 mM NaCl; 5 mM KCl; 0.7 mM Na_2HPO_4; 6 mM dextrose) were added to the DNA pellet. The DNA was allowed to resuspend in the 1X HeBS for at least two hours prior to electroporation. Immediately prior to electroporation, the DNA was pipetted up and down several times to mix.

[0103] Each electroporation procedure utilized approximately 2×10^7 DHFR⁻ CHO cells. These cells were fed or passaged on the day prior to electroporation and were approximately 50% confluent on 10 cm plates at the time of harvesting for electroporation. Cells were detached from the plates by trypsin treatment. The trypsin was subsequently inactivated by the addition of 8.0 ml α+ medium per plate. The contents of the plates were then centrifuged at 1000 rpm for 4 minutes in an IEC HN-SII table-top centrifuge (~200 X g_{av}) to pellet the cells. The medium was aspirated off of the cell pellet and the cells were resuspended in the remaining media by flicking the tube.

[0104] The plasmid and salmon sperm DNA (in 800 µl 1X HeBS) were added to the cell suspension and the mixture immediately transferred to an electroporation cuvette (Bio-Rad). The Bio-Rad Gene Pulser™ apparatus was used, with the capacitance set at 960 µFd and the voltage set at 300 V. To electroporate, one pulse was delivered to the contents of the cuvette at room temperature. No preincubation of the cell suspension/DNA mixture was required prior to the pulse. The duration of the pulse was approximately 10 milliseconds. The cells were incubated for 8-10 minutes at room temperature in the cuvette following electroporation and then were transferred to a 15-ml tube containing 14 ml of α⁺ medium. The cells were then centrifuged as above. After aspirating the medium, the wet cell pellet was resuspended by flicking, fresh α⁺ medium was added and the cells were pipetted gently up and down once or twice to resuspend further. The cells were then seeded into 10 cm plates. Approximately 20-30% of the viable CHO cells are expected to incorporate foreign DNA and, thus, survive primary selection. Therefore, approximately 1×10^7 total cells per 10 cm plate were seeded. To allow the cells to recover from electroporation, the cells were initially seeded into α⁺ non-selective medium.

[0105] Cells were cultured in a 37°C, 5.5% CO_2 incubator. After a recovery period of two days, the cells were removed from the culture plates by trypsin treatment, counted, and seeded into six 10 cm plates at a density of about 1×10^6 cells per plate, in α⁻ medium. The cells were incubated for four days in this primary selection medium (α⁻ medium). Approximately 15 - 30% of DHFR⁻ CHO cells were stably transformed to α⁻ resistance under these conditions, indicating that these cells had incorporated at least one copy of foreign DNA and were expressing the DHFR gene. After the four-day primary selection, the plates became nearly confluent with growing α⁻ resistant cells (α⁺ sensitive cells detach very slowly from the plates and after four days a large amount of sick, non-growing cells will remain attached).

[0106] After the four-day primary selection, the cells were subjected to secondary selection, to select for cells that had incoporated foreign DNA at a high copy number. The cells were again removed from the culture plates by trypsin treatment, counted, and seeded at a density of approximately 1×10^6 cells per plate into 10 cm plates containing α⁻/MTX medium (eight plates for each MTX concentration; see Tables I and II for the various MTX concentrations tested). After 6 - 7 days, the original medium was removed and the cells were fed with the fresh α⁻/MTX medium of the same composition. After 14 days of secondary selection, colonies (~1 mm diameter) were picked using a pipetman and seeded at one colony per well into 48-well tissue culture dishes.

[0107] In both experiments A and B, ten clones were picked after secondary selection for analysis at each MTX concentration, with the exception that at 0.5 µM MTX, twenty clones were picked from experiment A and twenty five from experiment B.

[0108] At confluence, the cells were transferred to 6-well dishes and later to 10 cm tissue culture plates. The cells were maintained in the same α⁻/MTX medium at all times.

[0109] The cells in 10 cm plates were fed with fresh α⁻/MTX medium at 50% confluence and were then detached by trypsin treatment. One-half of the cells was frozen down (overnight at -80°C) in 10% DMSO/90% α⁻ medium and then stored in liquid nitrogen until use. The other half of the cells was used as a source of genomic DNA for later Southern blot analysis to determine copy number.

Determination of DHFR Gene Copy Number

[0110] Nucleic acid was harvested from confluent or near-confluent 10 cm plates by removal of the culture medium followed by addition of 0.5 ml of a buffer containing proteinase K at 200 μg/ml in 0.5% sodium dodecyl sulfate/20 mM
5   Tris/HCl, pH 8/10 mM EDTA/10 mM sodium chloride. The resulting lysate was transferred to a 1.5 ml tube and incubated overnight at 37°C.

[0111] The nucleic acid was extracted twice with phenol, once with chloroform, and once with ether. Next, the nucleic acid was precipitated by the addition of sodium chloride to 0.1 M and 2.5 volumes of ethanol, followed by incubation for five minutes on dry ice. The nucleic acid was pelleted by a ten-minute centrifugation at 4°C in an Eppendorf centrifuge
10 (model 5414). The pellet was rinsed with -80°C ethanol, and then dried under vacuum. The nucleic acid was resuspended in 50 μl double-distilled water. Absorbance readings at 260 nm indicated yields of 50 - 100 μg of nucleic acid per 10 cm plate.

[0112] For analysis, 20 pg of nucleic acid was digested to completion (at least six hours, and often overnight, at 37°C) with PvuII and BglII. Digested DNA samples (20 μg nucleic acid per lane) were electrophoresed on 8% agarose gels
15 run in 1x TBE (see Maniatis et al., "Molecular Cloning: A Laboratory manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1980)). Each gel also contained several lanes of plasmid standards. The standards consisted of digested PvuII and BglII) pSV2-DHFR corresponding to various DHFR copy numbers of between 1 and 1000 copies per cell (usually corresponding to 2, 10, 50, 250, and 1000 copies). Assuming values of 3×10^9 base pairs per genome and 10 μg DNA per 20 μg nucleic acid (amount loaded per lane), we computed the amount of digested plasmid required
20 to give a single copy hybridization signal as being 16 pg pSV2-DHFR. This number was confirmed by comparison with hybridization signals of genomic DNA from clones known to contain only a single copy of pSV2-DHFR.

[0113] After electrophoresis, the nucleic acid in the agarose gels was transferred to Gene Screen™ hybridization membranes, using the protocol set forth in the Gene Screen™ manual (New England Nuclear, Boston, MA, #NEF-972). Pre-hybridization, hybridization, and washes were also performed according to the Gene Screen™ protocol.

25 **[0114]** The hybridization probe used was the ^{32}P-labelled 1000 bp PvuII/BglII fragment of pSV2-DHFR. The gel-purified fragment was labelled with ^{32}P according to the method of Feinberg and Vogelstein, "A Technique for Radiolabelling DNA Restriction Endonuclease Fragments to High Specific Activity", Anal. Biochem., 137, pp. 266-67 (1984).

[0115] After hybridization and washing (see above), the membranes were exposed to x-ray film at -80°C. Several films, exposed for differing times, were taken of each membrane. Copy number was then determined by comparing the
30 intensities of bands in the genomic DNA lanes with the same bands in the lanes containing the digested plasmid standards.

TABLE I

Effect of MTX Concentration on Frequency of Resistant Colonies		
[MTX]	Frequency of Resistant Colonies	
μM	A	B
0.1	7.5×10^{-5}	1.2×10^{-4}
0.2	1.9×10^{-5}	3.5×10^{-5}
0.3	1.1×10^{-5}	1.5×10^{-5}
0.4	6.3×10^{-6}	8.2×10^{-6}
0.5	4.1×10^{-6}	5.0×10^{-6}
1.0	2.2×10^{-6}	1.6×10^{-6}

TABLE II

Effect of MTX Concentration on DHFR Gene Copy Number				
[MTX]	Average Copy Number		Copy Number Range	
µM	A	B	A	B
0.1	8	6	4 - 20	2 - 14
0.2	14	17	8 - 25	10 - 25
0.3	31	18	15 - 40	4 - 25
0.4	41	60	8 - 120	6 - 250
0.5	113	109	15 - 500	10 - 800
1.0	106	92	20 - 400	25 - 300

* Ten clones were analyzed for copy number from each experiment at
MTX concentrations of 0.1, 0.2, 0.3, 0.4 and 1.0 µM. At 0.5 µM MTX,
20 clones from experiment A and 25 clones from experiment B were
analyzed.

EXAMPLE 2

Production of CHO Cells Containing the MIS Gene Integrated at High Copy Number

Cells and Media

[0116] The DHFR⁻ CHO cells described in Example 1 were also used for this example. The non-selective and primary selection media are the same as that described in Example 1. However, for secondary selection, all cells were cultured in α⁻ medium supplemented with 0.5 µM MTX.

Expression Vectors

[0117] The vector pJOD-10 (Figure 2) contains, as product gene, the human Müllerian inhibiting substance ("MIS") gene. The MIS gene is expressed from the Adenovirus 2 major late promoter ("AdMLP"), along with the SV40 enhancer. The MIS gene was derived from both cDNA and genomic DNA clones. Downstream from the MIS poly A addition site and 3'-genomic flanking sequence are SV40 splice and polyadenylation sites. This vector also contains the murine DHFR gene, derived from cDNA. The DHFR gene is expressed from the SV40 early promoter and is followed by SV40 splice and polyadenylation signals. The DHFR and MIS genes are expressed in opposite orientations. Between the two SV40 poly A sites is a transcriptional termination element. This element was synthesized as an oligonucleotide homologous to the human gastrin gene transcriptional termination sequence (see Sato et al., supra). This terminator element is employed in order to block transcriptional interference between the MIS and DHFR genes. The pJOD-10 vector also contains the ampicillin-resistance gene and the ColE1 bacterial origin of replication derived from pBR327, allowing cloning and amplification of this vector in bacteria.

[0118] Vector pJOD-10 was constructed (see Figure 2) from DNA of three origins: (1) vector PD1˙ (which comprises the human MIS gene); (2) vector pSV2-DHFR (which comprises the murine DHFR gene); and (3) synthetic oligonucleotide homologous to the human gastrin gene transcriptional terminator. The construction of PD1 is described in Cate et al., European patent application 221,761. The constructtion of vector pSV2-DHFR is described in Subramani et al., supra and is available from the American Type Culture Collection (ATCC 37146).

[0119] Two complementary oligonucleotides homologous to the human gastrin transcriptional terminator were synthesized according to standard procedures using an Applied Biosystems 380A DNA Synthesizer. These oligonucleotides were isolated by gel chromatography. The oligonucleotide corresponding to the gastrin gene coding strand is 51 nucleotides long and comprises a sequence homologous to nucleotides +190 to +233 of the human gastrin gene, according to the map coordinates and sequence of Sato et al., supra. The complementary oligonucleotide is 59 nucleotides long. These oligonucleotides were annealed, forming a double stranded DNA molecule ("term") with an APaI overhang at one end and an XhoI site and EcoRI overhang at the other end:

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        +190                                        +233
         |                                           |
5'       CCTTTTTTTTTTAATTTTTTATTTTTATTTTTATTTTTTGAGATGGAGTCTCGAGG    3'
3' CCGGGGAAAAAAAAAATTAAAAATAAAATAAAATAAAAACTCTACCTCAGAGCTCCTTAA 5'
```

[0120] Vector pSV2-DHFR was cut with EcoRI and Apal and the large fragment was gel purified. The double stranded term insert was then ligated into the Apal/EcoRI pSV2-DHFR fragment, forming vector pDT4. Vector pDT4 was cut with AatII and Xhol and the large fragment was gel purified. Vector pD1 was cut with Sall and AatII and the large fragment was gel purified. The Sall/AatII pD1 large fragment was inserted into the AatII/Xhol pDT4 large fragment, forming pJOD-10.

[0121] Vector pJOD-10, harbored in E. coli strain MC1061 was deposited in the In Vitro International, Inc. culture collection, in Linthicum, Maryland, on April 22, 1988. It is identified as "pJOD-10" and was assigned accession number IVI-10167. This deposit was transferred to the American Type Culture Collection, Rockyille, Maryland, U.S.A. on the 20 June, 1989, and has been assigned by them the accession number ATCC 68815.

Electroporation and Selection of High Copy Number CHO Cells

[0122] The protocol for production of CHO cells containing foreign DNA (pJOD-10) integrated at high copy number was the same as that followed in Example 1 except for the changes noted below.

[0123] Approximately 2×10^7 DHFR⁻ CHO cells were electroporated as described in Example 1 with a mixture consisting of 200 μg vector pJOD-10 and 200 μg sonicated salmon sperm DNA (as carrier). Vector pJOD-10 was linearized prior to electroporation by digestion overnight at 37°C with AatII. After a two-day recovery period in α^+ (non-selective) medium, the cells were seeded into α^- (primary selection) medium. The cells were cultured in this primary selection medium for four days. As in Example 1, approximately 15 - 30% of DHFR- CHO cells survived primary selection. Cells surviving primary selection were seeded into α^- medium supplemented with 0.5 μM MTX (the secondary selection medium). After 14 to 16 days of secondary selection, approximately fifty colonies remained, corresponding to a survival frequency of 4.2×10^{-6}. Twelve of these colonies were picked and expanded, preparatory to analysis. All twelve colonies survived expansion and were evaluated for MIS gene copy number and for MIS production level.

Determination of MIS Production Levels

[0124] MIS production levels were determined using the following sandwich ELISA protocol. Except where otherwise indicated, solutions added in washing steps filled the wells and other reagent solutions were added to the microtiter plate wells at a volume of 50 μl/well. Except where otherwise indicated, all incubations were performed at room temperature.

[0125] A solution containing monoclonal anti-MIS antibody M10.6 at a concentration of 25 μg/ml in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6 was added to each well of 96-well Dynatech Immulon II microtiter plates. The plates were then covered with Parafilm and incubated overnight to coat the wells. The next day, the contents of the plates were removed by shaking, and the plates were washed six times with water. Next, 150 μl of block buffer (5% fetal calf serum in phosphate buffered saline, filtered through 2 μm filter) were added to each well, and the plates were incubated for two hours.

[0126] After shaking out the blocking buffer, conditioned media (containing MIS), and several dilutions of conditioned media in block buffer, were added to the plates. Each plate included a row of dilutions of a solution containing pure MIS of known concentration (for standard curve) in block buffer, as well as a row of dilutions of block buffer alone (negative control). The plates were incubated for one hour.

[0127] The contents of the plates were then shaken out and the plates washed six times with water. Rabbit polyclonal anti-MIS Ab 848, at 1:1000 dilution in block buffer, was added to every well and the plates were incubated for one hour.

[0128] After shaking out the contents of the plate, the wells were washed four times with wash buffer (0.05% Tween 20 in phosphate buffered saline). Horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Kirkegaard & Perry Laboratories, Inc., Gaitherburg, MD, #041506), at 1:500 dilution in block buffer supplemented with 1% whole mouse serum (Cooper Biomedical, Inc., Malvern, PA, #5011-1380), was added to every well. The plates were incubated at 4°C for 50-60 minutes. After this incubation, the contents of the plates were shaken out and the plates were washed six times with wash buffer.

[0129] TMB/substrate buffer was added to every well and the development of blue color was monitored at 650 nm in a microtiter plate reader. After 10-15 minutes, color development was stopped by the addition to every well of 50 µl 2 N sulfuric acid. This addition changes the color of the solution from blue to yellow. Finally, after manually blanking the machine on one of the wells in the negative control row, the plate was automatically read at 450 nm.

[0130] TMB/substrate buffer was prepared immediately before use as follows: One milliliter of 42 mM tetramethylbenzidine (ICN Immunobiologicals, Lisle, IL, #980501) in dimethylsulfoxide (Aldrich) was added dropwise to 100 ml of room temperature 0.1 M sodium acetate/citric acid, pH 4.9. Lastly, 14.7 µl 30% hydrogen peroxide (Sigma Chemical Co. #H-1009) were added.

Determination of MIS Gene Copy Number

[0131] The MIS gene copy number of the twelve clones was determined analogously to the procedure followed in Example 1 for DHFR copy number, with the following modifications. In preparation for electrophoresis, nucleic acid isolated from the cells of this example was digested to completion with PvuII. For the plasmid standard lanes, pJOD-10 was likewise digested with PvuII. With respect to the plasmid standard lanes, the amount of digested pJOD-10 required to give a single copy hybridization signal was computed to be 33 pg. Finally, the hybridization probe in this example was the ^{32}P-labelled 1964 bp PvuII DNA fragment of pJOD-10 (pJOD-10 map coorindates 1854 to 3818).

TABLE III

HIGH COPY NUMBER ELECTROPORATION OF CHO CELLS WITH pJOD-10		
Clone	MIS production (pg/cell/day)	MIS gene copy number (copies per cell)
1	1.7	140
2	1.2	110
3	0.7	25
4	1.1	60
5	3.5	400
6	<0.1	10
7	0.8	50
8	0.5	100
9	6.0	700
10	0.3	40
11	<0.1	10
12	3.6	250

[0132] Table III displays the MIS gene copy number and the MIS production levels of the twelve clones picked for analysis. This data demonstrates that the process of this invention produces cells containing stably integrated foreign DNA at high copy number. The level of MIS production of all twelve clones was directly proportional to their MIS gene copy number. Eight of the twelve clones isolated have a high MIS gene copy number (over 50 copies/cell).

EXAMPLE 3

Production of 3T3 Cells Containing the MIS Gene Integrated at High Copy Number

Cells and Media

[0133] NIH/3T3 cells (ATCC CRL 1658) were propagated in DMEM (Gibco Laboratories) supplemented with 10% donor calf serum (Hazleton) and 4 mM glutamine ("DC medium"). For primary selection, the cells were cultured in DC medium, further supplemented with G418 (Gibco Laboratories) at 0.7 mg/ml ("DC-G medium"). For secondary selec-

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tion, the cells were cultured in DC medium supplemented with 0.5 µM MTX ("DC/MTX medium").

Expression Vectors

5 **[0134]** Protective (DHFR) and product (MIS) genes were supplied by vector pJOD-10 (see Figure 2). Vector pJOD-10 is described in Example 2. Vector pSV2-neo (Figure 3) contains the bacterial neo gene, which codes for G418-resistance in mammalian cells. The construction of pSV2-neo is described in Southern and Berg, "Transformation of Mammalian Cells to Antibiotic Resistance with a Bacterial Gene under Control of the SV40 Early Region Promoter", J. Mol. Appl. Genet., 1, pp. 327-41 (1982). The neo gene functions as a selective gene. In pSV2-neo, the neo gene is
10 expressed from the SV40 early promotor. Vector pSV2-neo, harbored in E. coli strain HB101, is available from the American Type Culture Collection (ATCC 37149).

Electroporation and Selection of High Copy Number 3T3 Cells

15 **[0135]** The protocol for production of NIH/3T3 cells (a DHFR$^+$ cell line) with high copy number integrated foreign DNA was the same as that followed in Example 1 for DHFR$^-$ CHO cells except for the changes noted below.
[0136] Approximately 2 x 10^7 3T3 cells were electroporated with a DNA mixture consisting of 220 µg pJOD-10, 40 µg pSV2-neo and 140 µg sonicated salmon sperm carrier DNA. Both the pJOD-10 and pSV2-neo vectors were digested overnight at 37°C with AatII to linearize them prior to electroporation. Electroporation was performed in a Bio-Rad Gene
20 Pulser™ by use of one pulse at 960 µFd and 270 V. After a 2-day recovery period in non-selective medium (DC medium), the cells were seeded at 6 x 10^5 cells per 10 cm plate into primary selection medium (DC-G medium). The primary selection step consisted of G418 selection for expression of the neo gene. The primary selection lasted 5 days, at the end of which approximately 5% of the 3T3 cells survived G418 selection. These cells were seeded, at 8 X 10^5 cells per 10 cm plate, into DC/MTX medium. After 14 days of secondary selection, eleven colonies remained, corre-
25 sponding to a secondary selection survival frequency of 8.5 x 10^{-7}. These colonies were picked and expanded as described in Example 1. Three colonies did not survive expansion. The MIS production level and the MIS gene copy number of the eight remaining colonies were determined as described in Example 2, and are displayed in Table IV.

30

TABLE IV

HIGH COPY NUMBER ELECTROPORATION OF 3T3 CELLS		
Clone	MIS production (pg/cell/day)	MIS gene copy number (copies per cell)
1	1.3	120
2	1.9	80
3	0.6	20
4	3.7	200
5	0.8	15
6	2.5	150
7	0.2	10
8	1.2	50

EXAMPLE 4

50 Gene Amplification After High Copy Number Electroporation

[0137] Clones 5, 9 and 12 of Example 2 are subjected to one round of DHFR/MTX amplification selection in α$^-$/MTX medium as described below. Culturing conditions and α$^-$ medium are described in Example 1.
[0138] To determine the appropriate MTX concentration for amplification of each of the clones, a MTX killing experi-
55 ment is performed as follows. Each parent clone is seeded at a density of 10^4 cells per 10 cm tissue culture plate in α$^-$ medium supplemented with MTX at the original concentration these clones were selected in (i.e., 0.5 µM MTX), and in α$^-$ medium supplemented with two-fold higher increments of MTX up to 10 µM MTX (i.e., 1.0, 2.0, 4.0, 8.0, and 10 µM MTX). After 7 days, the surviving cells of each clone are counted to determine the "maximum MTX surival concentra-

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tion" – the concentration which resulted in at least 50% as many cells surviving as those surviving at 0.5 μM MTX.

[0139] For amplification, each parent clone is seeded at a density of 5 x 10^3 cells per 10 cm plate in α⁻ medium supplemented with MTX at a concentration that is three-fold higher than the maximum MTX survival concentration for that parent clone. After 13 days of amplification selection, colonies derived from each parent clone are picked, expanded (in the same amplification selection medium) into 10 cm plates, and evaluated for MIS gene copy number and for MIS production level as described in Example 2.

[0140] The degree of gene amplification that will be produced by the amplification selection described above is variable. There apparently is an inverse relationship between the maximum MTX survival concentration of the parent clone, and its frequency of undergoing successful gene amplification. We do not as yet know the reason for this apparent inverse relationship.

[0141] The MIS gene copy number of those clones that are amplified by the above procedure is increased approximately 2 to 10-fold, while MIS production increases approximately 1.5 to 5-fold, from that observed in the parent clones.

Claims

1. A method for preparing recombinant host cells containing introduced foreign DNA at a copy number of 50 or greater, the method comprising the following steps:

    (a) subjecting an initial population of cells to electroporation in the presence of the introduced foreign DNA, wherein the introduced foreign DNA comprises:

       (i) at least one protective gene, coding for a protein capable of protecting the recombinant host cells against a toxic substance or decreases in the concentration of a previously essential nutritive substance in the culture medium, wherein increasingly higher copy numbers of the protective gene are required for and capable of protecting the recombinant cell against increasing concentrations of the toxic substance, or decreasing concentrations of the nutritive substance,
       (ii) at least one product gene coding for a polypeptide whose production is desired,

    the genes being operably linked to regulatory sequences that are compatible with the cells;
    (b) culturing the cells produced in step (a) for a time sufficient to allow these cells to recover from the electroporation process;
    (c) exposing the cells produced in step (b) to a sufficiently high concentration of the toxic substance, or a sufficiently low concentration of the nutritive substance, to kill or severely retard the division of those cells with a copy number of introduced foreign DNA of less than 50.

2. The method according to claim 1 wherein the protective gene codes for a protein capable of protecting the recombinant host cells against a toxic substance

3. The method according to claim 1 wherein the protective gene codes for a protein capable of protecting the recombinant host cells against decreases in the concentration of a previously essential nutritive substance in the culture medium.

4. The method according to claim 2 or 3, wherein the introduced foreign DNA in step (a) further comprises:

    at least one selective gene coding for a protein, wherein the presence of one copy per cell of the selective gene is sufficient to protect the recombinant host cells against either a decreased concentration of a second nutritive substance or the presence of a second toxic substance, said selective gene being operably linked to regulatory sequences that are compatible with the recombinant host cells,

    and wherein said method further comprises, before step (c), a step of exposing the cells produced in step (b) to either:

       (i) the toxic substance against which the product of said selective gene provides protection at a concentration sufficiently high to kill or severely retard the division of those cells which did not incorporate at least one copy of introduced foreign DNA in the electroporation process of step (a), or
       (ii) a decreased amount of the nutritive substance which the product of said selective gene makes non-essential in the context of the selection scheme at a concentration sufficiently low to kill or severely retard the division of those cells which did not incorporate at least one copy of introduced foreign DNA in the electroporation proc-

ess of step (a).

5. A method for improving production of the polypeptide whose production is desired in a recombinant host cell produced in the final step of any one of claims 1-4 comprising the step of culturing the cells.

6. The method according to any one of claims 1 to 5 wherein said protective gene is selected from the group of genes coding for dihydrofolate reductase, metallothionein, CAD, 3-hydroxy-3-methylglutaryl coenzyme A reductase, uridine monophosphate synthetase, adenosine deaminase, glutamine synthetase, asparagine synthetase, ribonucleotide reductase, thymidylate synthetase, omithine decarboxylase, or promoter glutathione S-transferase Ya.

7. The method according to claim 6, wherein said protective gene codes for dihydrofolate reductase.

8. The method according to any one of claims 1 to 5 wherein two or more of said protective product or selective genes of said introduced foreign DNA are contained within a single DNA molecule.

9. The method according to any one of claims 1 to 5 wherein said introduced foreign DNA is in the form of linear DNA molecules prior to electroporation.

10. The method according to claim 9, wherein said linear DNA molecules have compatible cohesive ends.

11. The method according to claim 8, wherein said DNA molecule further comprises at least one eukaryotic transcriptional terminator sequence located downstream of at least one of said genes.

12. The method according to any one of claims 1 to 5 comprising the further step of culturing the cells produced in the final step of any one of claims 1-5 in the presence of increasing amounts of the toxic substance or decreasing amounts of the nutritive substance which said protective gene was designed to protect against.

13. The method according to claim 12, wherein the protective gene codes for a DHFR capable of protecting the recombinant cells against MTX.

14. The method according to claim 13, wherein the concentration of the MTX is 2 to 10 fold greater than the maximum MTX survival concentration of the cells produced in the final step of any one of claims 1 to 5.

15. The method according to claim 14, wherein the concentration of MTX is three fold greater than the maximum MTX survival concentration of the cells produced in the final step of any one of claims 1 to 5.

16. The method according to claim 12 wherein the regulatory sequences operably linked to the protective and product genes are such that the protective gene is expressed at a lower level than the product gene.

17. The method according to claim 16, wherein the regulatory sequences cause the protective gene to be transcribed at a lower level than the product gene.

18. The method according to claim 16, wherein the regulatory sequences cause the protective gene messenger RNA to be less stable than the product gene messenger RNA.

19. A plural stage process, wherein stage one is the method according to any one of claims 1 to 5, and each subsequent stage is the method according to any one of claims 1 to 5, wherein the initial population of cells is the cells produced by the final step of the previous stage; and wherein the introduced foreign DNA used at each stage comprises a protective gene that is different from the protective gene used at any other stage.

20. The process according to claim 19, wherein there are two stages.

Patentansprüche

1. Ein Verfahren zum Herstellen rekombinanter Wirtszellen, die eingebrachte fremde DNA in einer Kopienzahl von 50 oder mehr enthalten, wobei das Verfahren die folgenden Stufen umfaßt:

(a) dem Unterwerfen einer anfänglichen Zellenpopulation einer Elektroporation in der Gegenwart der einge-

brachten fremden DNA, wobei die eingebrachte fremde DNA umfaßt:

(i) wenigstens ein Schutzgen, das für ein Protein kodiert, das in der Lage ist, die rekombinanten Wirtszellen gegen einen toxischen Stoff oder Abnahmen in der Konzentration eines vorher in dem Kulturmedium essentiellen Nährstoffes zu schützen, wobei zunehmend höhere Kopienzahlen des Schutzgens erforderlich und in der Lage sind, die rekombinante Zelle gegen ansteigende Konzentrationen des toxischen Stoffes oder abnehmende Konzentrationen des Nährstoffes zu schützen,
(ii) wenigstens ein Produktgen, das für ein Polypeptid, dessen Herstellung erwünscht ist, kodiert,

wobei die Gene mit regulatorischen Sequenzen, die mit den Zellen kompatibel sind, funktionsfähig verbunden sind;
(b) Kultivieren der in Stufe (a) hergestellten Zellen für eine ausreichende Zeit, sich diese Zellen von dem Elektroporationsverfahren erholen zu lassen;
(c) Exponieren der in Stufe (b) hergestellten Zellen einer ausreichend hohen Konzentration des toxischen Stoffes oder einer ausreichend niedrigen Konzentration des Nährstoffs, um die Teilung solcher Zellen mit einer Kopienzahl an eingebrachter fremder DNA von weniger als 50 stark zu verzögern oder zu töten.

2. Das Verfahren nach Anspruch 1, wobei das Schutzgen für ein Protein kodiert, das in der Lage ist, die rekombinanten Wirtszellen gegen einen toxischen Stoff zu schützen.

3. Das Verfahren nach Anspruch 1, wobei das Schutzgen für ein Protein kodiert, das in der Lage ist, die rekombinanten Wirtszellen gegen Abnahmen in der Konzentration eines vorher in dem Kulturmedium essentiellen Nährstoffes zu schützen.

4. Das Verfahren nach Anspruch 2 oder 3, wobei die eingebrachte fremde DNA in Stufe (a) weiter umfaßt:

wenigstens ein selektionierendes Gen, das für ein Protein kodiert, wobei die Gegenwart des selektionierenden Gens in einer Kopie pro Zelle ausreichend ist, um die rekombinanten Wirtszellen entweder gegen eine abnehmende Konzentration eines zweiten Nährstoffes oder die Gegenwart eines zweiten toxischen Stoffes zu schützen, wobei das selektionierende Gen mit regulatorischen Sequenzen, die mit den rekombinanten Wirtszellen kompatibel sind, funktionsfähig verbunden ist,

und wobei das Verfahren vor der Stufe (c) weiter eine Stufe des Exponierens der in Stufe (b) hergestellten Zellen umfaßt, entweder:

(i) dem toxischen Stoff, gegen den das Produkt des selektionierenden Gens Schutz liefert, in einer ausreichend hohen Konzentration, um die Teilung solcher Zellen, die nicht wenigstens eine Kopie der in dem Elektroporationsverfahren von Stufe (a) eingebrachten fremden DNA einbauten, stark zu verzögern oder zu töten, oder
(ii) einer verringerten Menge des Nährstoffes, den das Produkt des selektionierenden Gens im Zusammenhang mit dem Selektionsschema nichtessentiell macht, in einer ausreichend niedrigen Konzentration, um die Teilung solcher Zellen, die nicht wenigstens eine Kopie der in dem Elektroporationsverfahren von Stufe (a) eingebrachten fremden DNA einbauten, stark zu verzögern oder zu töten.

5. Ein Verfahren zum Verbessern der Herstellung des Polypeptids, dessen Herstellung erwünscht ist, in einer in der letzten Stufe nach einem der Ansprüche 1-4 hergestellten rekombinanten Wirtszelle, das die Stufe des Kultivierens der Zellen umfaßt.

6. Ein Verfahren nach einem der Ansprüche 1 bis 5, wobei das Schutzgen ausgewählt wird aus der Gruppe von Genen, die für Dihydrofolatreduktase, Metallothionein, CAD, 3-Hydroxy-3-methylglutaryl Coenzym A Reduktase, Uridinmonophosphatsynthetase, Adenosindesaminase, Glutaminsynthetase, Asparaginsynthetase, Ribonukleotidreduktase, Thymidylatsynthetase, Ornithindecarboxylase, oder Promotor-Glutathion S-transferase Ya kodieren.

7. Das Verfahren nach Anspruch 6, wobei das Schutzgen für Dihydrofolatreduktase kodiert.

8. Das Verfahren nach einem der Ansprüche 1 bis 5, wobei zwei oder mehr der Schutz-, Produkt- oder selektionierenden Gene der eingebrachten fremden DNA in einem einzigen DNA-Molekül enthalten sind.

9. Das Verfahren nach einem der Ansprüche 1 bis 5, wobei die eingebrachte fremde DNA vor der Elektroporation in der Form von linearen DNA-Molekülen vorliegt.

10. Das Verfahren nach Anspruch 9, wobei die linearen DNA-Moleküle kompatible kohäsive Enden haben.

11. Das Verfahren nach Anspruch 8, wobei das DNA-Molekül weiter wenigstens eine eukaryotische transkriptionale Terminatorsequenz, die abwärts von wenigstens einem der Gene liegt, umfaßt.

12. Das Verfahren nach einem der Ansprüche 1 bis 5, umfassend die weitere Stufe des Kultivierens der in der letzten Stufe nach einem der Ansprüche 1 bis 5 hergestellten Zellen in der Gegenwart ansteigender Mengen des toxischen Stoffes oder abnehmender Mengen des Nährstoffes, gegen die zu schützen das Schutzgen entwickelt wurde.

13. Das Verfahren nach Anspruch 12, wobei das Schutzgen für eine DHFR kodiert, die in der Lage ist, die rekombinanten Zellen gegen MTX zu schützen.

14. Das Verfahren nach Anspruch 13, wobei die Konzentration des MTX 2 bis 10 mal größer ist als die maximale MTX-Überlebenskonzentration der in der letzten Stufe nach einem der Ansprüche 1 bis 5 hergestellten Zellen.

15. Das Verfahren nach Anspruch 14, wobei die Konzentration an MTX dreimal größer ist als die maximale MTX-Überlebenskonzentration der in der letzten Stufe nach einem der Ansprüche 1 bis 5 hergestellten Zellen.

16. Das Verfahren nach Anspruch 12, wobei die regulatorischen Sequenzen mit den Schutz- oder Produktgenen funktionsfähig verbunden sind, so daß das Schutzgen auf einem niedrigerem Niveau als das Produktgen exprimiert wird.

17. Das Verfahren nach Anspruch 16, wobei die regulatorischen Sequenzen bewirken, daß das Schutzgen auf einem niedrigerem Niveau als das Produktgen transkribiert wird.

18. Das Verfahren nach Anspruch 16, wobei die regulatorischen Sequenzen bewirken, daß die Schutzgen-Boten-RNA weniger stabil als die Produktgen-Boten-RNA ist.

19. Ein mehrstufiges Verfahren, wobei Stufe Eins das Verfahren nach einem der Ansprüche 1 bis 5 ist, und

jede folgende Stufe das Verfahren nach einem der Ansprüche 1 bis 5 ist, wobei die anfängliche Zellenpopulation die durch die letzte Stufe der vorherigen Stufe hergestellten Zellen sind; und wobei die eingebrachte fremde in jeder Stufe verwendete DNA ein Schutzgen, das von dem in einer anderen Stufe verwendeten Schutzgen verschieden ist, umfaßt.

20. Das Verfahren nach Anspruch 19, wobei es dort zwei Stufen gibt.

Revendications

1. Méthode de préparation de cellules hôtes recombinées contenant un ADN étranger introduit à un nombre de copies d'au moins 50, cette méthode comprenant les étapes suivantes:

(a) on soumet une population initiale de cellules à une électroporation en présence de l'ADN étranger introduit, l'ADN étranger introduit comprenant:

(i) au moins un gène protecteur codant pour une protéine capable de protéger les cellules hôtes recombinées contre une substance toxique ou contre des réductions de la concentration d'une substance nutritive auparavant essentielle dans le milieu de culture, des nombres de copies croissants du gène protecteur étant nécessaires et aptes à la protection de la cellule recombinée contre des concentrations croissantes de la substance toxique ou des concentrations décroissantes de la substance nutritive,

(ii) au moins un gène de produit codant pour un polypeptide dont on désire la production,

les gènes étant liés de façon opérationnelle à des séquences régulatrices qui sont compatibles avec les cellules;

(b) on cultive les cellules produites dans l'étape (a) pendant un temps suffisant pour permettre à ces cellules de se remettre de l'opération d'électroporation;

(c) on expose les cellules produites dans l'étape (b) à une concentration suffisamment élevée de la substance toxique ou à une concentration suffisamment basse de la substance nutritive pour tuer les cellules ayant un nombre de copies d'ADN étranger introduit inférieur à 50 ou en retarder sérieusement la division.

2. Méthode selon la revendication 1, dans laquelle le gène protecteur code pour une protéine capable de protéger les cellules hôtes recombinées contre une substance toxique.

3. Méthode selon la revendication 1, dans laquelle le gène protecteur codes pour une protéine capable de protéger les cellules hôtes recombinées contre des réductions de la concentration d'une substance nutritive auparavant essentielle dans le milieu de culture,

4. Méthode selon la revendication 2 ou 3, dans laquelle l'ADN étranger introduit dans l'étape (a) comprend en outre:

au moins un gène sélectif codant pour une protéine, la présence d'une copie par cellule du gène sélectif étant suffisante pour protéger les cellules hôtes recombinées contre une concentration réduite d'une seconde substance nutritive ou contre la présence d'une seconde substance toxique, ledit gène sélectif étant lié de façon opérationnelle à des séquences régulatrices compatibles avec les cellules hôtes recombinées,

et ladite méthode comprend en outre, avant l'étape (c), une étape d'exposition des cellules produites dans l'étape (b)

(i) soit à la substance toxique contre laquelle le produit dudit gène sélectif fournit une protection, à une concentration suffisamment élevée pour tuer les cellules n'ayant pas incorporé au moins une copie d'ADN étranger introduit dans l'opération d'électroporation de l'étape (a) ou en retarder sérieusement la division,

(ii) soit à une quantité réduite de la substance nutritive que le produit dudit gène sélectif rend non essentielle dans le contexte du schéma de sélection, à une concentration suffisamment faible pour tuer les cellules n'ayant pas incorporé au moins une copie d'ADN étranger introduit dans l'opération d'électroporation de l'étape (a) ou en retarder sérieusement la division.

5. Méthode pour améliorer la production du polypeptide dont on désire la production dans une cellule hôte recombinée produite dans l'étape finale de l'une quelconque des revendications 1 à 4, comprenant l'étape de culture des cellules.

6. Méthode selon l'une quelconque des revendications 1 à 5, dans laquelle ledit gène protecteur est choisi dans le groupe des gènes codant pour la dihydrofolate-réductase, la métallothionéine, la CAD, la 3-hydroxy-3-méthyl-glutaryl-coenzyme A-réductase, l'uridine-monophosphate-synthétase, l'adénosine-désaminase, la glutamine-synthétase, l'asparagine-synthétase, la ribonucléotide-réductase, la thymidylate-synthétase, l'ornithine-décarboxylase ou la glutathion-S-transférase de promoteur Ya.

7. Méthode selon la revendication 6, dans laquelle ledit gène protecteur code pour la dihydrofolate-réductase.

8. Méthode selon l'une quelconque des revendications 1 à 5, dans laquelle deux ou plusieurs desdits gènes protecteurs, gènes de produit ou gènes sélectifs dudit ADN étranger introduit sont contenus dans une seule molécule d'ADN.

9. Méthode selon l'une quelconque des revendications 1 à 5, dans laquelle ledit ADN étranger introduit est sous forme de molécules d'ADN linéaires avant l'électroporation.

10. Méthode selon la revendication 9, dans laquelle lesdites molécules d'ADN linéaires ont des extrémités cohésives compatibles.

11. Méthode selon la revendication 8, dans laquelle ladite molécule d'ADN comprend en outre au moins un terminateur de transcription eucaryote situé en aval d'au moins l'un desdits gènes.

12. Méthode selon l'une quelconque des revendications 1 à 5, comprenant l'étape supplémentaire selon laquelle on cultive les cellules produites dans l'étape finale de l'une quelconque des revendications 1 à 5 et 9 en présence de

quantités croissantes de la substance toxique ou de quantités décroissantes de la substance nutritive contre les-quelles ledit gène protecteur doit fournir une protection.

13. Méthode selon la revendication 12, dans laquelle le gène protecteur code pour une DHFR capable de protéger les cellules recombinées contre le MTX.

14. Méthode selon la revendication 13, dans laquelle la concentration du MTX est de 2 à 10 fois supérieure à la concentration de MTX maximale de survie des cellules produites dans l'étape finale de l'une quelconque des revendications 1 à 5.

15. Méthode selon la revendication 14, dans laquelle la concentration de MTX est trois fois supérieure à la concentration de MTX maximale de survie des cellules produites dans l'étape finale de l'une quelconque des revendications 1 à 5.

16. Méthode selon la revendication 12, dans laquelle les séquences régulatrices liées de façon opérationnelle au gène protecteur et au gène de produit sont telles que le gène protecteur est exprimé à un niveau inférieur à celui du gène de produit.

17. Méthode selon la revendication 16, dans laquelle les séquences régulatrices font que le gène protecteur est transcrit à un niveau inférieur à celui du gène de produit.

18. Méthode selon la revendication 16, dans laquelle les séquences régulatrices font que l'ARN messager du gène protecteur est moins stable que l'ARN messager du gène de produit.

19. Procédé en plusieurs stades, dans lequel le premier stade est constitué par la méthode selon l'une quelconque des revendications 1 à 5

et chacun des stades successifs est constitué par la méthode selon l'une quelconque des revendications 1 à 5, la population initiale de cellules étant formée par les cellules produites par l'étape finale du stade précédent ; et
l'ADN étranger introduit dans chaque stade comprenant un gène protecteur différent du gène protecteur utilisé dans l'un quelconque des autres stades.

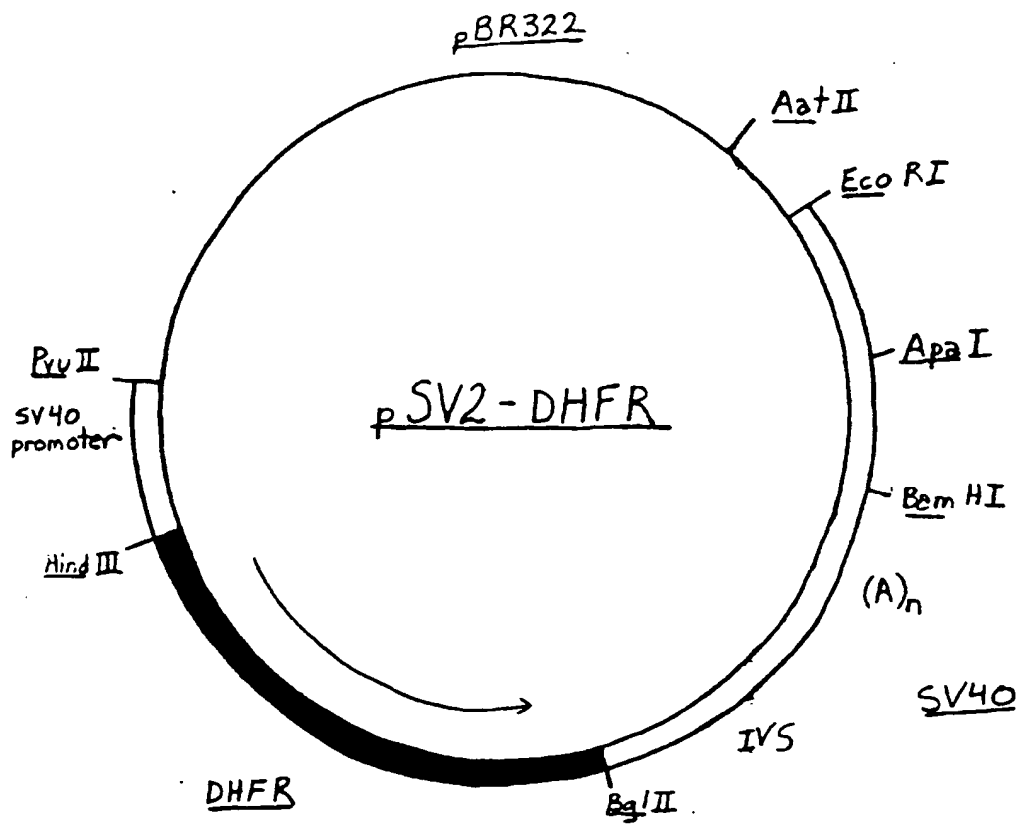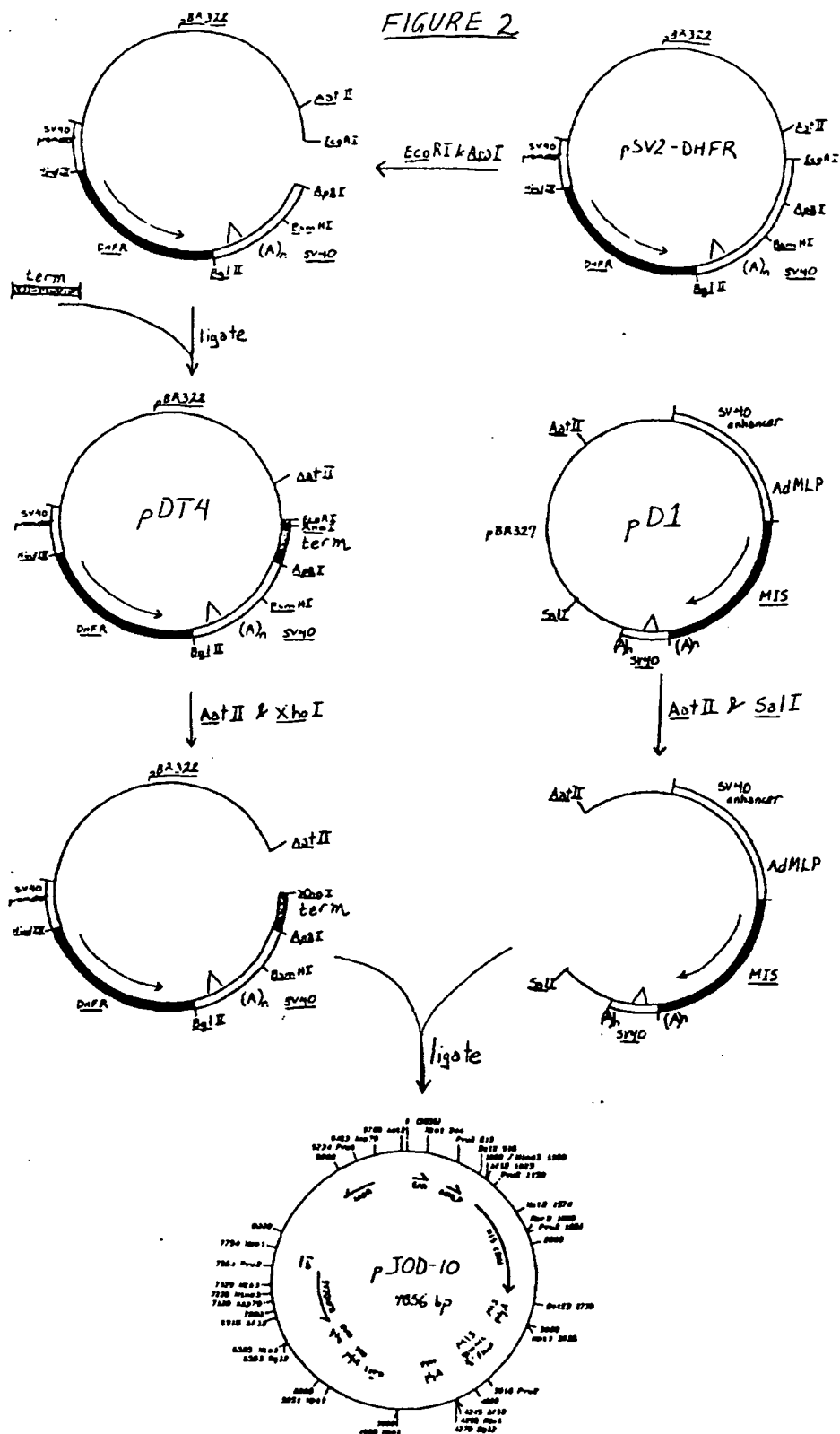20. Procédé selon la revendication 19, qui comprend deux stades.

FIGURE 1

FIGURE 2

FIGURE 3